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Alpha (v) integrins license regulatory T cells to apoptotic cells and self-associated antigens

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Defects in apoptotic cell clearance are thought to contribute to autoimmunity by failure to induce tolerance, coupled with accumulation of immunogenic material. However, little is known about the contribution of apoptosis to immune responses at mucosal sites, where regulatory T cells (T_{reg} cells) and other immune cells play an essential active role in maintaining tolerance to self-associated antigens. In recent studies, we have found that α_v integrins have an important role in apoptotic cell phagocytosis and induction of T_{reg} cells in the intestine, and deletion of α_v from myeloid cells causes colitis associated with failed apoptotic cell removal and loss of T_{reg} cells. Our data show that activation of transforming growth factor (TGF)-β by $\alpha_v\beta_8$ on dendritic cells (DCs) is essential for generating T_{reg} cells and inducing mucosal tolerance. These results provide a mechanism by which tolerance to apoptotic cell–derived and –associated antigens is maintained by DC "licensing" at sites of high TGF-β expression.

Keywords: integrins; apoptosis; phagocytosis; TGF-β; regulatory T cells; colitis

Introduction

Cell death is a normal part of life in a multicellular organism, helping to sculpt organs during development and partnering with cell proliferation in tissue homeostasis and renewal. This process is generally regulated and orderly, with cells undergoing a series of characteristic changes, including cell shrinkage, DNA degradation, and surface reorganization, a process termed *programmed cell death* or *apoptosis*. Dying cells will eventually break up into membrane-bound vesicles or "apoptotic bodies." *In vivo*, dying cells or resulting apoptotic bodies are swiftly removed by neighboring phagocytic cells with little or no inflammation or tissue perturbation. Consequently, homeostatic apoptosis is rarely evident in uninflamed tissues. This rapid removal is critical, as there is increasing evidence that the persistence of uncleared apoptotic cells can lead to inflammation and autoimmunity.[1] Hence, phagocytosis can be considered an essential partner of apoptosis in a multicellular organism, ensuring that the dying cell is removed and digested.

Cell death and the subsequent removal of apoptotic cells by phagocytosis is an evolutionarily ancient process and the central components of apoptosis, recognition, phagocytosis, and digestion pathways are conserved between the nematode *Caenorhabditis elegans,* the fruit fly *Drosophila melanogaster,* mice, and humans. Apoptotic cells are recognized by changes on their cell surface, caused by oxidation or modification of existing surface molecules, by expression of new molecules, and by loss of normal surface components of living cells. The best-characterized apoptotic cell surface component is phosphatidylserine (PS), which is normally confined to the inner leaflet of the plasma membrane, but is rapidly externalized during apoptosis. Through PS and other surface changes, receptors on neighboring cells or specialized phagocytes are able to bind apoptotic cells, leading to engulfment and digestion.[2] Engagement of

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apoptotic cell receptors also triggers specific signals in phagocytes, including the peroxisome proliferator-activated receptor (PPAR)-δ, PPAR-γ, and liver X receptor (LXR) pathways,[3–6] and causes release of anti-inflammatory mediators, which include the immunomodulatory cytokines transforming growth factor (TGF)-β and interleukin 10 (IL10).[7–10] Hence, unlike the phagocytosis of microbes or components of cell damage, which trigger inflammatory cytokine production, apoptotic cells generally suppress inflammatory responses and promote immune tolerance, resolution of inflammation, and tissue regeneration.

The apparent simplicity of apoptotic cell removal belies a complex process, involving many surface and soluble receptors, which probably reflect the importance of this process to metazoans. In mammals, the number of putative "apoptotic cell receptors" continues to grow, encompassing a wide range of molecules (see, for example, the recent review by Nagata in Ref. 1). Many of these receptors can be classified into relatively simple classes: soluble components of the complement and collectin cascade (including C1q and mannose binding lectin [MBL]); scavenger receptors and lectins (including CD36, scavenger receptor A [SRA1], multiple epidermal growth factor-repeats [MEGF]-10 and CD14); receptors that bind PS directly (T cell immunoglobin domain and mucin domain protein [Tim1–4], brain-specific angiogenesis inhibitor 1 [BAI-1], Stabilin 3); receptor tyrosine kinases that bind PS indirectly through opsonins (MerTK with Protein S); and integrins, that bind apoptotic cell surface components through various soluble proteins. However, many of these receptors are not solely involved in apoptotic cell uptake, and have wider roles in innate immunity. As an example, scavenger receptors such as CD36 bind a range of oxidized lipids and other self-associated molecules, as well as microbial components, which they present to Toll-like receptors (TLRs) to trigger inflammatory signals. Similarly, complement components are central to innate and adaptive immune responses and host defense to infection and injury. How these molecules combine to distinguish homeostatic cell death from that occurring in the context of infection, tissue damage or malignancy, and initiate appropriate responses, remains poorly understood. Although we currently we do not have a full understanding of how these receptors work together, there is some evidence that different phagocytic cells, particularly macrophages and dendritic cells (DCs), utilize distinct receptors. It is also becoming apparent that certain tissues preferentially use certain phagocytic pathways, particularly in sites of high cell turnover such as lymphoid organs, skin, retina, and the testis. As we discuss below, the response to apoptotic cells and the immunological outcomes are likely to reflect the microenvironmental and cellular context, as much as the individual receptors engaged by the dying cell.

Alpha (v) integrins: receptors for apoptotic cells

Our interest has been in one major family of apoptotic cell receptors, the α_v integrins, or vitronectin receptor family. Alpha (v) integrins comprise a family of five αβ heterodimers ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$) with overlapping ligand specificities and functions. $\alpha_v\beta_3$ was the first apoptotic cell receptor to be identified,[11] and along with the closely related $\alpha_v\beta_5$, has been shown to mediate phagocytosis of apoptotic cells and related particles by many different cell types, including the professional phagocytes of the immune system, macrophages and DCs.[12,13] We have shown that macrophages from α_v, β_3 or β_5 knockout mice have deficient phagocytosis of apoptotic cells *in vitro*[10,14] and that α_v-knockout macrophages and DCs clear apoptotic cells less efficiently *in vivo*.[14] In a rare example of results from human studies being subsequently validated by genetic analyses in model organisms, integrins were recently shown to participate in apoptotic cell phagocytosis in *C. elegans*, demonstrating that this mechanism of apoptotic cell phagocytosis is likely to be evolutionarily ancient.[15]

Integrins α_v bind apoptotic cells via secreted molecules such as thrombospondin (TSP),[16] matrix protein fragments, developmental endothelial locus (Del)1 and milk fat globule (MFG)-E8.[17,18] Although the ligands for TSP and matrix proteins on the apoptotic cell surface have not been identified, Del1); and MFG-E8 have been shown to bind aminophospholipids including PS to promote engulfment.[17,18] MFG-E8 is expressed at high levels by macrophages and DCs, Langerhans cells, and follicular DCs, and appears to be of particular importance for apoptotic cell removal by macrophages in lymphoid organs. Mice deficient in MFG-E8 accumulate apoptotic cells in the germinal centers and

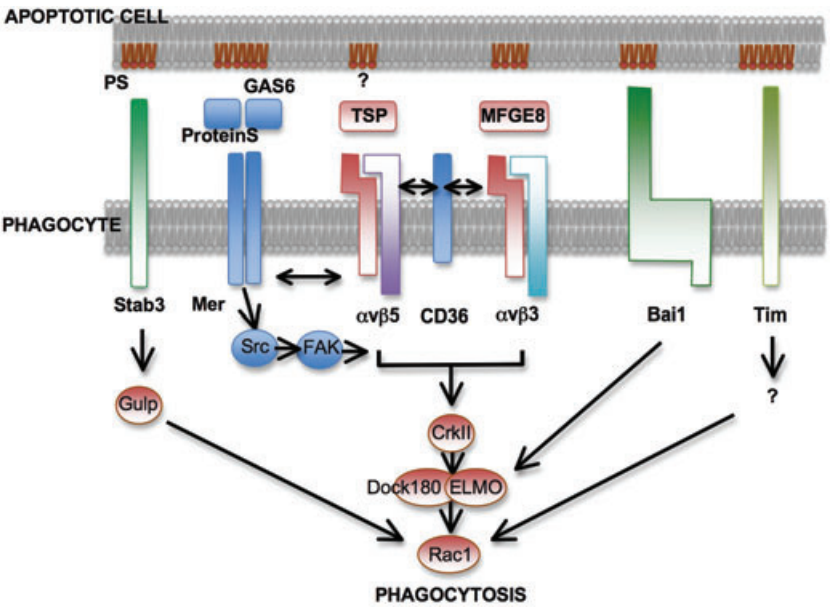

Figure 1. **Molecules implicated in the recognition of phosphatidylserine (PS) during apoptotic cell phagocytosis. PS is recognized through a direct interaction with receptors (e.g., Tim-1, Tim-3, and Tim-4, Bai1 and stabilin 3) or through soluble bridging molecules, such as Protein S, GAS6, and MFGE8, coupled to Mer or α_v integrins. Thrombospondin (TSP) also bridges apoptotic cells to α_v integrins and CD36, although the ligand for TSP on apoptotic cells is unknown. Also shown are reported interactions of apoptotic cell receptors with α_v-mediated internalization pathways, which culminate in activation of Rac, cytoskeletal reorganization, and phagocytosis.**

develop a lupus-like autoimmunity, underscoring the apparent importance of apoptotic cell removal in lymphoid organs and implicating MFG-E8, and hence αv, as critical components of that process.[19]

Integrins interact closely with the cytoskeleton through their cytoplasmic domains, and upon ligand binding, promote cytoskeletal changes and adhesion or migration. Similar mechanisms allow integrins to initiate efficient membrane reorganization during phagocytosis, through a cascade including p130Cas, CrkII, Dock180, and Elmo, eventually causing activation of the small GTPase Rac1.[20] Integrins can also undergo complex conformational changes in response to intracellular signals, permitting rapid changes in affinity and avidity for their ligands, which then trigger rapid cytoskeletal reorganization.[21] It is likely, therefore, that apoptotic cell binding to various nonintegrin receptors will activate α_v or other integrins, which in turn trigger phagocytosis, a mechanism termed *tether and tickle* when first proposed in the context of the putative PS receptor, PSR.[22] Supporting this model, there is evidence that α_v integrins cooperate with several different receptors to mediate phagocytosis (Fig. 1). In the first studies of α_v-mediated phagocytosis, α_v on macrophages was found to cooperate with the scavenger receptor CD36.[23] Similar α_v-CD36 mechanisms were shown to operate in DCs and other nonprofessional phagocytes[12,13,24] and to trigger intracellular signaling to activate Rac1,[20] although the mechanism by which CD36 and α_v cooperate has not been defined.

The receptor tyrosine kinase Mer has also been shown to cooperate with α_v to mediate apoptotic cell uptake.[25] Mer, and related kinases Tyro and Axl, bind apoptotic cells through serum proteins (Protein S and growth arrest specific [GAS]-6), which recognize exposed PS on apoptotic cells. Following apoptotic cell binding, Mer activates Src, which recruits focal adhesion kinase (FAK); to $\alpha_v\beta_5$, promoting engulfment through Rac1.[25] This mechanism appears to be particularly important in epithelial cells at sites of high tissue turnover such as the retina, where outer segments of photoreceptor neurons must be cleared in large numbers daily. The relative simplicity of this system, with a single apoptotic particle: phagocyte pair, highly synchronized phagocytosis, and well-defined outcome make this an elegant system to dissect this process *in vivo*. In beautiful studies, it has been

shown that phagocytosis of shed neuron segments occurs through $\alpha_v\beta_5$/Mer/CD36, with cross talk between β_5 and Mer mediated by FAK,[26,27] although results from this system place Mer downstream of integrin signaling.[28] Deletion of Mer or β_5 both lead to blindness; however, whereas loss of Mer causes failed clearance, retinal degeneration and early blindness, β_5 knockouts instead develop late onset blindness associated with accumulation of autofluorescent storage bodies in the retinal pigment epithelium. It appears that although basal phagocytosis can occur in the absence of β_5, the synchronization of photoreceptor uptake through $\alpha_v\beta_5$ and FAK is essential for efficient degradation of internalized material.[28] Whether α_v integrins are similarly involved in efficient processing of apoptotic material in antigen presenting cells remains to be determined.

Alpha (v) integrins regulate mucosal immunity

Alpha (v) integrins therefore serve as multifunctional apoptotic cell receptors, contributing to phagocytosis in different organs and numerous cell types, and interacting with several distinct recognition receptors. This central role of α_v in phagocytosis of apoptotic cells and other cell debris prompted us to study the role of these integrins in this process *in vivo*. As α_v-knockout mice are neonatally lethal, because of placental and developmental defects, we developed a conditional knockout allele (α_v-flox mice) to allow deletion in specific cell populations.[14,29] Crossing these to tie2-CRE and LysM-CRE transgenics generated mice lacking α_v in all hematopoietic cells (α_v-tie2), or in myeloid cells (α_v-LysM), respectively.[14] Both conditional knockout lines were viable, with no obvious developmental defects, and survived into adulthood. Phagocytosis in the peritoneal cavity, and *ex vivo* by macrophages and DCs derived from these mice, was impaired, consistent with our previous *in vitro* studies of β_3- and β_5-knockout mice. Furthermore, α_v-tie2 and α_v-LysM mice developed high titers of anti-DNA and anti-nuclear antibodies, a phenotype seen with several other apoptotic cell receptor knockouts, including the α_v-dependent bridging molecule MFG-E8.[19] Together, these data support a central role for α_v integrins in phagocytosis of apoptotic cells, and subsequent promotion of immune tolerance.

Both lines of mice also developed spontaneous inflammation at mucosal surfaces particularly in the colon and lung.[14] This was associated with an increase in activated T cells and expression of high levels of Th1 and Th2 cytokines, suggesting that inflammation was caused by defects in T cell regulation. Consistent with this, α_v-tie2 and α_v-LysM mice had lower numbers of regulatory T cells (T_{reg} cells, expressing CD4, CD25, and the transcription factor FoxP3) in the intestine. Regulatory lymphocytes, including T_{reg} cells, are required for control of mucosal immune responses and loss of these cells causes T cell activation and intestinal inflammation. T_{reg} cells themselves subdivide into two populations that differ in their specificity and effector mechanisms: "natural" T_{reg} (nT_{reg}) cells and "induced" or "adaptive" T_{reg} (aT_{reg}) cells.[30] nT_{reg} cells develop in the thymus as a polyclonal pool that recognize many self-antigens, make up the majority of $CD4^+$ $FoxP3^+$ cells in the spleen and lymph nodes (LNs); and regulate activation of self-reactive T cells and prevent autoimmunity.[30] In contrast, aT_{reg} cells are generated from mature T cells in the periphery, and suppress immune responses to self- and nonself antigens in tissues and lymph nodes. The ability to induce T_{reg} cells from naive T cell populations seems to be of particular importance in the intestine; unlike defects in nT_{reg} cells that lead to widespread autoimmunity and inflammation, defects in aT_{reg} cells lead primarily to intestinal inflammation. Selective loss of aT_{reg} cells is therefore the likely cause of colitis in α_v-deficient mice.

Importantly, the loss of aT_{reg} cells and development of colitis occurred when α_v was deleted specifically in myeloid cells, and was therefore due to defects in macrophages and DCs, rather than primary defects in T cells. A specific subpopulation of intestinal DCs, which express the integrin $\alpha_E\beta_7$ (CD103), are required for generation of aT_{reg} cells in mice,[31] and both CD103 expression and generation of aT_{reg} cells are dependent on TGF-β.[31–36] In α_v-tie2 mice $CD103^+$ DCs were decreased in number and were less able to generate T_{reg} cells *in vitro*, implicating defects in TGF-β signaling in this phenotype. TGF- β is synthesized as an inactive latent precursor, which must be activated by proteolysis or conformational change before it can signal. α_v integrins, particularly $\alpha_v\beta_6$ and $\alpha_v\beta_8$, can bind and activate latent TGF-β and concomitant with our studies, similar findings were reported for β_8-integrin conditional knockout

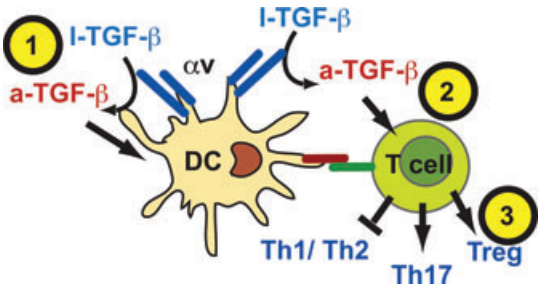

Figure 2. Model of role for $\alpha_v\beta_8$ integrin in TGF-β activation by DCs. (1) DCs bind and activate latent TGF-β (l-TGF-β) through $\alpha_v\beta_8$ integrin, which promotes generation of "regulatory" DCs. (2) Regulatory DCs bind, activate, and present active TGF-β (a-TGF-β) to T cells to suppress Th1 and Th2 responses and stimulate generation of aT_{reg} and/or Th17 cells. (3) aT_{reg} cells maintain immune tolerance and homeostasis.

mice.[37] With these findings, we and others have proposed that $\alpha_v\beta_8$ expressed by DCs locally activates TGF-β, which acts in a paracrine fashion to initiate T_{reg} cell differentiation in T cells (Fig. 2).[14,37] Furthermore, our data suggest that TGF-β production by T cells is insufficient to regulate immunity in the absence of TGF-β activation by DCs, which serve to "license" TGF-β responses. Such a model would provide a mechanism by which antigen specificity could be regulated and retained in T_{reg} cells in a TGF-β-rich environment such as the intestine. α_v is also likely to contribute to TGF-β signaling to DCs during their conditioning in the intestine, and expression of one marker of "regulatory DCs," α_E integrin, is itself regulated by TGF-β.

On the basis of our data, we propose a model in which α_v integrins have a dual role in mucosal tolerance: $\alpha_v\beta_3$ and $\alpha_v\beta_5$ mediate uptake of apoptotic cells by macrophages and DCs, thus providing self antigen and modifying the extent of inflammation; and $\alpha_v\beta_8$ generates T_{reg} cells through local activation of TGF-β and conditioning of DCs (Fig. 3). Although these processes use different β subunits, it is tempting to speculate that they are closely linked and interrelated *in vivo*, particularly as many of the effects of apoptotic cells have been attributed to the release of TGF-β and generation of T_{reg} cells.

TGF-*β* production and T_{reg} and Th17 cell differentiation

The uptake of apoptotic cells by macrophages and DCs directly stimulates the synthesis and release of TGF-β.[8–10,38] This, in turn, has been shown to suppress inflammatory responses by macrophages,

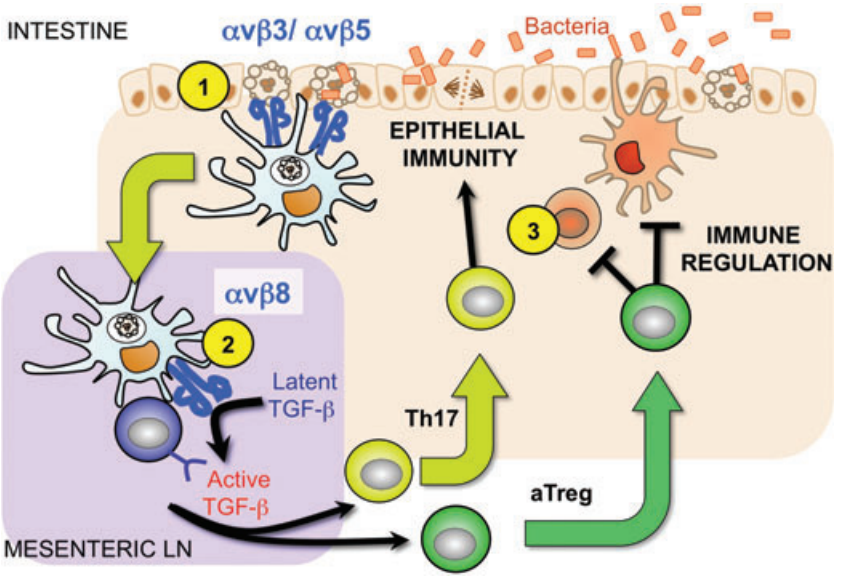

Figure 3. Dual role for α_v integrins in mucosal immune regulation. (1) $\alpha_v\beta_3$ and $\alpha_v\beta_5$ mediate uptake of apoptotic epithelial cells by macrophages and DCs in the intestinal lamina propria, providing self and self-associated antigen; (2) $\alpha_v\beta_8$ activates TGF-β locally during interactions with antigen-specific T cells, "licensing" TGF-β signaling in T cells, and promoting generation of aT_{reg} and Th17 cells; (3) aT_{reg} and Th17 cells regulate immune responses in the intestinal lamina propria and promote epithelial barrier immunity, respectively.

stimulate resolution of inflammation and initiate immune tolerance through generation of T_{reg} cells.[8,39,40] $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have been implicated in TGF-β production, either directly[41] or by association with CD36 or PS-mediated apoptotic cell phagocytosis.[39,42] We would predict that $\alpha_v\beta_8$ (or possibly other α_v integrins) would also be required for efficient activation of apoptotic cell–stimulated TGF-β and generation of T_{reg} cells. Although this has not yet been proven directly, there is indirect evidence from several studies consistent with a requirement for an additional TGF-β activation step in regulatory responses to apoptotic cells. For example, in our studies, the presence of TGF-β in the culture medium was not sufficient to inhibit cytokine production in macrophages and apoptotic cell contact was required for the full immunosuppressive effects of apoptotic cells.[10] It is possible that the process of apoptotic cell recognition, via cytoskeletal reorganization or other signaling pathways, causes activation or upregulation of α_v, promoting TGF-β activation and permitting immunosuppressive signaling. Furthermore, although apoptotic cell phagocytosis promotes T_{reg} cell generation through TGF-β production, both macrophages and DCs seem to be required for this to occur efficiently *in vivo*.[40,43] We speculate that this may be because TGF-β can be produced by macrophages, other bystander cells, or even delivered on the apoptotic cell itself, whereas β_8 integrin, which is required for TGF-β activation during T_{reg} cell generation, is more tightly regulated and is limited to DCs.[37]

Apoptotic cell phagocytosis and resulting TGF-β production have also been implicated in the differentiation of a second lineage of T helper cells, Th17 cells. Initially characterized by the production of IL17A and IL17F, Th17 cells are now known to produce a number of proinflammatory and immunoregulatory cytokines, including IL21 and IL22, and are defined by the expression of the transcription factor ROR-γT. Th17 cells orchestrate immune responses to extracellular bacteria and fungi at mucosal surfaces and are most prevalent in the intestinal lamina propria, where they are generated in response to microbial colonization.[44,45] Th17 cells share a common precursor with aT_{reg} cells, and differentiation of both lineages requires TGF-β. However, whereas TGF-β alone is sufficient to promote T_{reg} cell generation, TGF-β combined with proinflammatory cytokines such as IL6 or IL1β promotes Th17 cells, which are further expanded by IL23. In a recent study, Torchinsky *et al.*[46] showed that DCs exposed to both apoptotic cells and bacterial stimulation produce both TGF-β and proinflammatory cytokines and stimulate Th17 generation. Furthermore, intestinal infection with *Citrobacter rodentium*, a pathogenic bacterium, caused a Th17-dominated immune response that was dependent on widespread apoptosis in the intestinal epithelium. This study established the important concept that apoptosis in the context of an infectious challenge may not lead directly to immune tolerance, but instead to generation of Th17 responses.

Apoptotic cell clearance at mucosal surfaces

The more widespread consequences of Th17 responses to apoptotic cell–derived antigens are still to be established, in part because the exact contributions of Th17 cells to immunity and inflammation remain unclear. Th17 cells are present at high numbers in mucosal tissues, and increase in number following challenge with pathogens. Their normal function is therefore thought to be mediating defense against potential mucosal pathogens through stimulation of innate immune responses. However, Th17 cells are also found at high numbers in the intestine in the absence of inflammation, suggesting an additional role in intestinal immune homeostasis. In support of this, Th17 cells are known to secrete IL22, which stimulates epithelial repair and protects against intestinal inflammation. Th17 cells may therefore serve to promote the integrity of the epithelial barrier, in addition to orchestrating more direct immune responses against microbes (Fig. 3).[47–49] This would be particularly beneficial in the intestine, where the presence of large numbers of microbes are tolerated by the action of an effective epithelial barrier, rather than engagement of more "conventional" inflammatory responses. It is also intriguing that T_{reg} and Th17 cells share a common lineage, and are both generated in response to apoptotic cells. We have previously speculated that T_{reg} cells generated in response to apoptotic cells serve to maintain immune tolerance to apoptotic antigens and prevent immune-mediated destruction[14,38]; Th17 cells may likewise promote epithelial integrity to prevent cellular damage caused by intestinal microbes, particularly microbes that adhere

tightly to the epithelium, such as segmented filamentous bacteria or *Citrobacter*, which also triggers apoptosis.[44,46] It will be informative to study the contributions of T_{reg} and Th17 cells in intestinal immunity in more detail, particularly in the context of epithelial cell death. The α_v-conditional knockout mice should be particularly instructive in this regard as our unpublished data suggest that Th17 responses are also impaired in these mice.

Many previous studies of the effects of apoptotic cell clearance on immunity have focused on effects on immune and autoimmune responses in lymph organs. The data discussed above on α_v-deficient mice and Th17 development suggest that apoptotic cell clearance also has an important role in initiating appropriate immune responses at mucosal sites, particularly the intestine. Indeed, phagocytosis of apoptotic cells has previously been linked to immune regulation in the intestine. Analysis of DCs trafficking from the intestine to the mesenteric lymph nodes identified a population of DCs with distinct surface markers (OX41/signal-regulatory protein [SIRP]α in the rat) that contained remnants of apoptotic intestinal epithelial cells.[50] These apoptotic cell–bearing DCs were present even in the absence of intestinal inflammation, expressed low levels of costimulatory molecules and were poor stimulators of T cell proliferation.[50,51] Such "functionally immature" DCs preferentially induce T_{reg} cells when compared with classically activated DCs and it is therefore likely that apoptotic cell–containing DCs are responsible for generation of aT_{reg} cells in the mesenteric lymph nodes. It is striking that DCs of a similar phenotype can be induced *in vitro* by culture with apoptotic cells.[38] These data raise the interesting question of whether the regulatory DC phenotype is intrinsic to subpopulations of DCs, as has been suggested for $CD103^+$ intestinal DCs, or is induced by phagocytosis of apoptotic cells, as can occur in culture *in vitro*. In all likelihood, we expect that the truth probably lies between these two extremes; DC subsets that contribute to regulatory cell development appear to be specialized for apoptotic cell uptake, and in these DCs, apoptotic cells promote the production of TGF-β and other cytokines required for T_{reg} cell generation.

Summary

Recent studies have highlighted the importance of apoptotic cell clearance at mucosal surfaces and link apoptotic cell recognition by DCs to induction of immunoregulatory T cells and a specialized effector population, Th17 cells. Our results from α_v-deficient mice provide an important mechanism by which tolerance to apoptotic cell–derived and associated antigens is maintained by DC "licensing" at sites of high TGF-β expression such as the intestine. Furthermore, these findings suggest that the induction of apoptosis by microbes may be beneficial in establishing immune networks that enable immune defense against pathogens whilst permitting microbial colonization. We have set out a model in which phagocytosis of apoptotic cells provides intestinal DCs with both antigen and appropriate conditioning to generate T_{reg} and Th17 cells. We propose that α_v integrins have a dual role in mucosal tolerance: (1) $\alpha_v\beta_3$ and $\alpha_v\beta_5$ mediate uptake of apoptotic cells by macrophages and DCs, thus providing self and self-associated antigen and stimulating release of TGF-β; (2) $\alpha_v\beta_8$ activates TGF-β during interactions with antigen-specific T cells, promoting generation of T_{reg} and Th17 cells.

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Conflicts of interest

The authors declare no conflicts of interest.

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